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Botulinum Type A Toxin: Properties of a Toxic Dissociation Product

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Introduction

This paper will be devoted to a description of the properties of a substance which makes its appearance when solutions of type A botulinum toxin are brought to pH 7.5. At this pH value the greater part of the toxin forms a diffuse, rapidly sedimenting boundary in the ultracentrifuge, while about 14% sediment much more slowly (7). Partial separation of this slowly sedimenting component having been accomplished in the preparative rotor of the ultracentrifuge (7) it became possible to compare its properties with those of the parent substance. Among the results to be reported here, particular significance must be ascribed to the demonstration that the slowly sedimenting component represents a fully active form of the toxin which is free from the hemagglutinin commonly associated with it.

Materials and Methods

Botulinum Toxin

Two specimens of toxin were used in the experiments reported below. These, which will be designated preparations *e* and *f*, were both obtained from a single batch of toxin solution purified by the method of Abrams, Kegeles, and Hottle (1). Preparation *e* was the material precipitated at 5°C. by 0.8 *M* ammonium sulfate and Prepn. *f* was obtained when the ammonium sulfate concentration in the mother liquor from Prepn. *e* was increased to 1.2 *M*. Both preparations were crystalline. In the analytical ultracentrifuge, a 1.6% solution of Prepn. *e* in 0.05 *M* acetate buffer, pH 3.8, formed a sharp boundary of sedimentation constant, s^{w}_{20} of 9.5 which accounted for at least 95% of the sedimenting material while the remaining 5% or so appeared as a faint boundary which moved much more rapidly. Under

[1] The material herein presented will form part of a forthcoming Ph.D. thesis by Jack Wagman, Georgetown University, Washington, D. C.

similar conditions a 1.2% solution of Prepn. *f* showed a major boundary of sedimentation constant 12.5, together with subsidiary boundaries with sedimentation constants 17.4 and 11.7 representing somewhat larger proportions of the sedimenting material than the subsidiary component in Prepn. *e*. In each of these preparations, the value of s^{w}_{20} for the main boundary was in good agreement with data given by Wagman and Bateman (7) on the concentration dependence of the sedimentation constant for a monodisperse preparation at pH 3.8–4.0, which, by linear extrapolation of $1/s$, yielded a value of 16.5 for s^{w}_{20} at zero concentration. When brought to pH 7.5 by dialysis against 0.05 *M* phosphate buffer, the two preparations had the properties summarized in Table I. Since the presence of a slowly sedimenting component was established, in confirmation of the results reported

TABLE I

Sedimentation Data for Fractions of Botulinum Type A Toxin Formed at pH 7.5

Preparation[a]	Concn.[b]	*c* rel.			$10^{13}\, s^{w}_{20}$		
	g./100 ml.	%			*sec.*		
e							
1 Original	0.520	15	78	7	7.3	21.8	29.2
2 Upper-half supernatant	0.038	100	—	—	—[c]	—	—
3 Lower-half	0.118	68	32	—	6.9	19.7	—
4 Redissolved pellet	0.586	9	82	9	6.9	21.4	25.4
f							
1 Original	0.400	25	75	—	5.6	21.7	—
2 Upper-half supernatant	0.075	100	—	—	3.7	—	—
3 Lower-half	0.206	91	9	—	4.7	18.7	—
4 Redissolved pellet	1.493	8	92	—	7.4	18.9	—

[a] For key to preparations see text, "*Materials and Methods.*"

[b] Determined refractometrically, using specific refractive index increment 1.86×10^{-3}.

[c] Concentration too low and boundary too diffuse for calculation of *s*.

by Wagman and Bateman (7) for another toxin preparation, it was appropriate to proceed with fractionation of Prepns. *e* and *f* in the preparative rotor.

Ultracentrifugal Fractionation

The toxin solutions, after having been brought to pH 7.5 by dialysis against 0.05 *M* phosphate buffer, were centrifuged for about 3 hr. in the preparative rotor of the model E Spinco ultracentrifuge at an average field strength of $150{,}000 \times g$. The toxin concentration was about 1%, the volume centrifuged was 10 ml., and the average temperature was 20°C. After centrifuging, the supernatant solution was divided into upper and lower halves, and the gelatinous pellet was redissolved in phosphate buffer, pH 7.5.

Sedimentation and Diffusion Studies

Sedimentation constants were determined using the analytical rotor of the model E Spinco ultracentrifuge. The records were analyzed for sedimentation constants and relative concentrations of components. Diffusion was measured at 3.2°C. in a Claesson cell (4) with the schlieren optical system, and diffusion constants were calculated by the second-moment method.

Electrophoresis

Electrophoretic mobilities were measured in the Klett type of Tiselius apparatus, using the standard form of cell and a bath temperature of 3.2°C.

Other Measurements

Ultraviolet absorption was measured in the Beckman type DU spectrophotometer using the 1-cm. cell.

Specific areas of monolayers of toxin were measured by spreading the toxin solution on 3% NaCl in the small film tray described previously (2) and measuring the area at a film pressure of 13 dynes/cm., using an "indicator oil" end point.

Paper chromatographic amino acid analysis of the hydrolyzed fractions was performed by the method of Housewright and Thorne (5) using both phenol and aqueous ethanol as solvents.

Toxicity for mice was determined by intraperitoneal injection of diluted toxin into 18–20 g. white mice. The toxin was diluted with 1% disodium phosphate buffer, pH 6.8, containing 0.2% gelatin. The volume injected was 0.5 ml. and six mice were used for each dilution. The LD_{50} was defined as the dose, in mg. N, which killed half the mice within 4 days. If dilutions are made in small steps in the region of the end point, the LD_{50} can probably be determined with an accuracy of 10–20%.

Hemagglutination titers were read by the Lush pattern method as described by Burnet (3), using chick red cells.[2]

Flocculation titers were determined using a crude antitoxic plasma (Lederle).

EXPERIMENTAL

Characterization of Fractionated Toxin in the Ultracentrifuge

The fractionation procedure described above when applied to Preps. *e* and *f* afforded six specimens. The success with which the fractionations were carried out is attested by the result of examining the six specimens in the ultracentrifuge. In both experiments reported in Table I the upper half of the solution contained the slowly sedimenting component, in rather low concentration, virtually free from the rapidly sedimenting material that constituted nearly 80% of the starting solution. The lower half of the solution contained the slowly sedimenting component con-

[2] *Erratum.* In the previous paper by the present authors (7) it was erroneously stated that sheep red cells were used for hemagglutinin assay. Chick cells were used throughout.

taminated with 32% of heavy material in one case and with 9% in the other. The solutions made by dissolving the pellets contained 9 and 8%, respectively, of the slowly sedimenting component.

A marked progressive increase in the sedimentation constant of the light component of Prepn. *f* in passing from the upper layer of solution to the pellet provides further evidence of the polydisperse state of this component. There will also be noted in the same experiment discrepancies between the sedimentation constants of the heavy component of the various fractions. It should be mentioned in this connection that at pH 7.5 the solutions become rather turbid and are clarified by centrifuging prior to the performance of an analytical run in the ultracentrifuge or of

TABLE II

Sedimentation and Diffusion Data for Polydisperse Slowly Sedimenting Fraction Formed at pH 7.5 (Dissociated Toxin)

Measurement made in 0.05 *M* phosphate buffer (ionic strength 0.13) (Solution *f*3, Table I)

Sedimentation constant, $10^{13}s^{w}_{20}$ (*sec.*)	4.7[a]
Diffusion constant, $10^{7}D^{w}_{20}$ (*sq. cm./sec.*)	6.74
Partial specific volume, $\bar{V}$ (assumed)	0.76
Molecular weight, M[c]	71,000
Frictional ratio, f/f_0	1.14
Axial ratio, a/b[b,c]	3.4

[a] For a solution of concentration 0.206 g./100 ml.

[b] For an elongated ellipsoid of revolution.

[c] The values of M, f/f_0, and a/b are provisional since they are based on measured values of s and D and not on extrapolated values.

any other test. Since the rapidly sedimenting component is polydisperse, precipitation would perhaps consist in preferential removal, by aggregation, of the heaviest molecules, thus bringing about a displacement of the distribution curve of sedimentation rates in the direction of a decreased average sedimentation constant. This may account for the tendency of the average sedimentation constant of the heavy component to decrease during the course of an experiment.

Sedimentation and Diffusion Data on Slowly Sedimenting Fraction

Only one of the solutions (*f*3, Table I) was suitable for measurement of a diffusion constant. The results of this measurement, together with the data derived from it, are given in Table II. The average molecular weight, 71,000, for the slowly sedimenting component is probably some-

what too high, since the measured value of the diffusion constant may have been influenced by the presence of 9 % of the heavy component. The value is also open to slight correction when sufficient data become available for extrapolation of s and D to zero concentration.

Other Physicochemical Properties of Fractions

Solution *f*3 contained two electrophoretically resolvable components in phosphate buffer (0.04 *M* phosphate + 0.1 *M* NaCl) at pH 7.4. The mobility of the main boundary was -3.52×10^{-5} sq. cm./v. sec., that of the secondary boundary, corresponding to about 5 % of the nondialyzable material, was -14.6×10^{-5}. The ultraviolet absorption spectrum of the solution was consistent with the hypothesis that the secondary boundary in the electrophoresis experiment was formed by nucleic acid, since the absorption maximum occurred at 258 mμ, while that of the unfractionated toxin occurred at 278 mμ, in the position characteristic of most proteins.

Chromatographic analysis revealed no apparent differences in the proportions of aspartic acid, glutamic acid, leucine (and/or isoleucine), valine, and phenylalanine present in the various fractions.

The original toxin preparations and the various fractions all formed insoluble films when applied to the surface of a 3 % NaCl solution. The specific areas at 13 dynes/cm. of films formed from the slowly sedimenting material (0.38–0.45 sq. m./mg.) were significantly greater than those formed from the original preparations (0.23 sq. m./mg.) or from the redissolved sediments (0.22, 0.18 sq. m./mg.).

Toxicity, Hemagglutination and Flocculation Titers

The biological tests performed upon the toxin fractions and upon the starting material are reported in Table III. The results of the experiments on Preps. *e* and *f* are concordant in the sense that while indicating that the toxicities of all the fractions are of the same order of magnitude as those of the starting materials, great differences exist between the hemagglutinating activities of the fractions. The hemagglutinin titers given for the fractions from which the rapidly sedimenting component had been largely removed are rough estimates only, but the values suggest that the hemagglutination brought about by these fractions is due solely to the heavier contaminant, and that the slowly sedimenting material is devoid of hemagglutinating activity.

The relatively small differences between the toxicities of the various solutions show some inconsistencies, for the data on Prepn. *e* would sug-

gest that the toxicity is equally distributed throughout the protein nitrogen of the various fractions, while in the case of Prepn. *f* it would seem that the slowly sedimenting component is significantly more toxic than the starting material or the sedimented pellet. Inasmuch as the fractionation resulted in a greater degree of separation of the components in the latter case, greater weight should be given to the conclusion suggested by this experiment.

Ramon flocculation tests were performed only upon the material from Prepn. *f*. It will be seen from the results in Table III that when the *Lf* values of the various fractions are expressed in terms of the toxicities of the fractions (in LD_{50} units), there are no significant differences between them. The same is true when the nitrogen content serves as the unit of

TABLE III

Properties of Botulinum Type A Toxin Fractions

Preparation:	*e*				*f*			
Fraction:[a]	1	2	3	4	1	2	3	4
Toxicity, $10^{-6} \times LD_{50}$/mg. N	190	197	186	214	155	220	200	135
Hemagglutination, $10^{-3} \times LD_{50}$/ml.[b]	8	64	32	8	6	66	30	3
Hemagglutination, $10^{5} \times$ mg. N/ml.	4.21	32.5	17.2	3.74	3.87	30.0	15.0	2.22
Flocculation titer, $Lf/10^{6}LD_{50}$	—	—	—	—	3.0	2.7	2.0	3.4
Flocculation titer, *Lf*/mg.N	—	—	—	—	470	590	400	460
Flocculation time, *min.*	—	—	—	—	75	620	90	49

[a] Key to fractions: 1 is starting material.
2 is upper half of solution after centrifuging for about 3 hr. at 150,000 × *g*.
3 is lower half of solution.
4 is solution of sedimented material.

[b] Chick erythrocytes at 1°C.; concentrations required for detectable agglutination.

concentration. However, this method of reporting conceals the fact that the slowly sedimenting material flocculated with the antiserum only at relatively high *absolute* concentrations. Fractions *f*2 and *f*3 gave no flocculation when the quantities used were identical with those of fractions *f*1 and *f*4 that had been found to give satisfactory end points. A six- to tenfold increase was needed in order to carry out a satisfactory titration with fractions *f*2 and *f*3, and even under these circumstances the flocculation time was greatly prolonged.

Discussion

Properties of Dissociated Toxin

The experiments reported in this and in the earlier paper (7) have shown that at pH 7.5 type A botulinum toxin exists in the form of two

clearly distinguishable polydisperse components, one of average molecular weight of the order of magnitude of one million, the other at least one order of magnitude smaller. Although the range of molecular weights existing about these two average values is unknown, it is probably justifiable to assume that there is no significant overlap, and to treat the two components as though they were distinct entities with certain properties in common and with certain important differences.

In the following discussion the rapidly sedimenting polydisperse component at pH 7.5 will be referred to as the "*complex toxin*," the slowly sedimenting polydisperse component as the "*dissociated toxin*," while the material of molecular weight of about one million that exists below pH 4 in monodisperse form in the best preparations (and with some paucidispersity in others) will be designated "*paucidisperse toxin*." These names have the merit that they avoid any commitment as to whether the polydispersity of the "complex toxin" is one of molecular shape or of size.

In certain respects the complex toxin and the dissociated toxin appear to be alike. They were not distinguishable on the basis of their contents of several amino acids. The electrophoretic mobility of the dissociated toxin, -3.52×10^{-5} sq. cm./v. sec. at pH 7.4, is consistent with the pH mobility curve recorded by Abrams, Kegeles, and Hottle (1) for what was then regarded as the pure toxin, while the presence of a secondary boundary which can be attributed to free nucleic acid explains the observed differences between the ultraviolet absorption spectra of the various fractions prepared by us. The nucleic acid is clearly in the uncombined state and its presence in the "dissociated" or monomeric form of toxin preparation probably means that a trace of nucleic acid attached to the original material at pH values below 4 becomes released at pH 7.5.

In toxicity, the dissociated toxin resembles the parent material, although perhaps significantly more toxic. On the other hand, the hemagglutinin titers indicate that the type A toxin can exist in a form which is not capable of agglutinating red cells. Whether, conversely, the agglutinin can be obtained in nontoxic form has not been shown by the present experiments. This possibility seems however to be indicated by the observation (6*a*) that the toxicity of toxin solutions is not decreased when the solutions have been used to agglutinate red cells. Recently Lamanna and Lowenthal (6*b*) have brought immunological evidence that botulinus antitoxin preparations contain two components, an antitoxin and an antihemagglutinin, of which the latter is relatively nonspecific. Finally, unpublished experiments in this laboratory by M. S. Davis and P. A.

McCaffrey show conclusively that the hemagglutinin is removed from toxin solutions when red cells are agglutinated, without bringing about any measurable change in the sedimentation diagram of the toxin.

The relationships between the various forms of the toxin are by no means clear. The toxicities/mg. N being almost equal, it would seem reasonable to suppose that the paucidisperse form found at pH values below 4 consists of an assembly of the smaller toxin molecules which at some stage during the course of physiological action of the toxin becomes transformed to the complex toxin and then completely dissociated into its components; these may tentatively be identified with the fundamental toxic units, although the experimental evidence does not preclude the possibility that the toxic unit is still smaller than the molecule of dissociated toxin. The complete dissociation has not yet been observed in the laboratory; at pH 7.5 it occurred only to the extent of about 20% under the conditions of our experiments, even when several days were allowed for the process. This may represent complete dissociation of 20% of the original material, the extent of dissociation being limited either by the attainment of an equilibrium which favors the complex toxin, or by other unrecognized factors. Alternatively, it may mean that, on the average, 20% of the toxic units in the monodisperse toxin are more loosely bound than the remainder; the latter hypothesis would seem to be the more plausible.

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Summary

1. The partial dissociation of botulinus type A toxin at pH 7.5 and ionic strength 0.13 into a slowly sedimenting component has been confirmed.

2. Under the above conditions of pH and ionic strength the sedimentation constant of the undissociated material is about 20 svedberg units, while that of the dissociated portion is about 5 (see Table I). Both are polydisperse.

3. Measurements of sedimentation and diffusion constants of a specimen of the "dissociated toxin" separated in the ultracentrifuge led to values of 71,000 for the molecular weight and 3.4 for the axial ratio.

4. The toxicity of the "dissociated toxin" formed at pH 7.5 is at least as great, per milligram protein nitrogen, as that of the parent substance. The flocculation titer is also the same. The hemagglutinin titer is very low, probably essentially zero.

5. The nonhemagglutinating polydisperse "dissociated toxin" of molecular weight around 70,000 is identified tentatively with the ultimate toxic unit of botulinus type A toxin.

References

1. Abrams, A., Kegeles, G., and Hottle, G. A., *J. Biol. Chem.* **164**, 63–79 (1946).
2. Bateman, J. B., *J. Cellular Comp. Physiol.* **29**, 85–9 (1947).
3. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.* **20**, 81–8 (1942).
4. Claesson, S., *Nature* **158**, 834 (1946).
5. Housewright, R. D., and Thorne, C. B., *J. Bacteriol.* **60**, 89–100 (1950).
6. (*a*) Lamanna, C., *Proc. Soc. Exptl. Biol. Med.* **69**, 332–6 (1948); (*b*) Lamanna, C., and Lowenthal, J. P., *J. Bacteriol.* **61**, 751–752 (1951).
7. Wagman, J., and Bateman, J. B., *Arch. Biochem. and Biophys.* **31**, 424–30 (1951).